



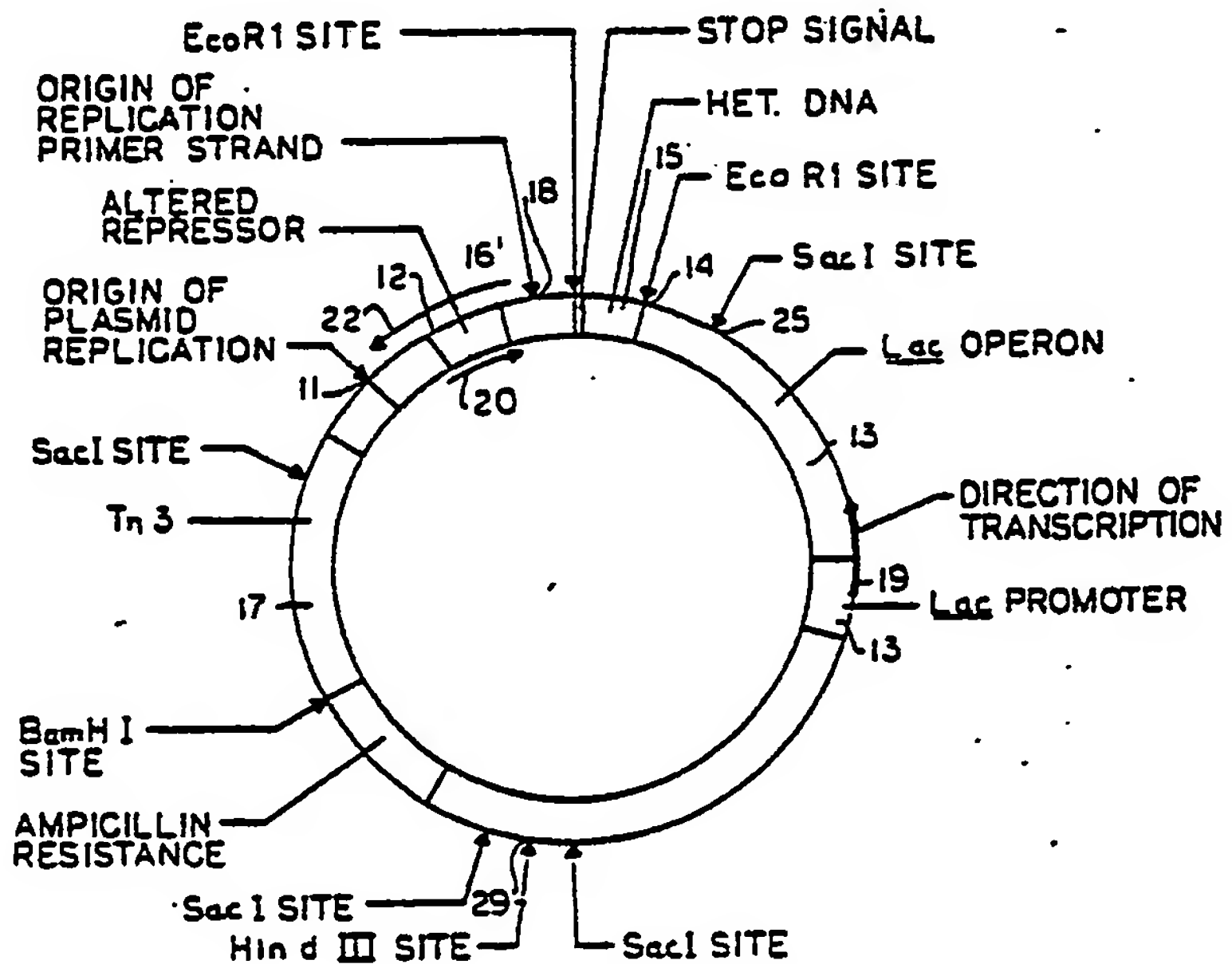
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification³ : C12N 15/00, 1/00; C12P 21/00 C12P 21/02, 21/04; C07G 7/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 82/ 02901 (43) International Publication Date: 2 September 1982 (02.09.82)</p>
<p>(21) International Application Number: PCT/US82/00246 (22) International Filing Date: 1 March 1982 (01.03.82) (31) Priority Application Number: 239,165 (32) Priority Date: 27 February 1981 (27.02.81) (33) Priority Country: US (71) Applicant: CETUS CORPORATION [US/US]; 600 Bancroft Way, Berkeley, CA 94710 (US). (72) Inventor: GELFAND, David, Harow ; 6208 Chelton Drive, Oakland, CA 94611 (US). (74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin, Flannery & Welsh, Room 900, 135 South La Salle Street, Chicago, IL 60603 (US).</p>		<p>(81) Designated State: AU. Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>

(54) Title: STABLE HIGH COPY NUMBER PLASMIDS

(57) Abstract

DNA plasmids which are selected mutants in which an altered repressor gene leads to high copy number replication. Elements in the plasmids are modified by placing a suitable transcriptional 'stop' signal after an inserted heterogenous gene so that readthrough expression of heterologous DNA inserted in the plasmid will not continue into the replication primer strand. Deletions resulting from interference with replication primer strand transcription are thereby avoided.



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STABLE HIGH COPY NUMBER PLASMIDS

This invention relates to molecular biology and, more particularly to a technique for increasing the levels of expression of protein products encoded by heterologous DNA in cloning vectors.

Genetic change can occur randomly as a result of mutations appearing in a gene. As a result of a change in the gene, a corresponding change may occur in the protein which it encodes, changing the resultant properties of the organism. With the advent of recombinant DNA techniques, such genetic changes may be made deliberately by the introduction of a known nucleotide sequence from one strain or species into another. The known nucleotide sequence may be selected to confer a desired property upon the strain or species into which it is introduced. When the modified strain or species proceeds with the normal replication process, it also then duplicates the inserted sequence.

A commonly used recombinant DNA technique involves breaking open the double-stranded DNA of a plasmid cloning vector at a desired location where foreign DNA is to be inserted. To do this, particular types of proteins, called restriction enzymes, are typically used. Certain restriction enzymes will break the DNA at particular nucleotide sequences. If two different types of DNA are severed in a similar manner, the open ends will therefore be complementary and will, under suitable conditions, stick together with the complementary ends lying side-by-side. They may then be linked together enzymatically (with ligase). This makes it possible to insert or "splice" a foreign DNA segment from any source into the desired location in the plasmid cloning vector.

All DNA, whether from microbes or from complex plants or animals, consists of the same identical set of nucleotides. Thus, when a DNA fragment derived from a



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foreign source is spliced into a plasmid, and the plasmid is introduced into a suitable host microorganism, the replication system of the host reproduces the inserted segment along with the DNA of
5 the original host.

Once in the host, the foreign or heterologous DNA is not only replicated from generation to generation, but also will produce protein for which it is encoded. This assumes the proper reading frame and
10 promoters exist. The amount of protein produced by heterologous DNA as a result of recombination depends, of course, on the magnitude and efficiency of the process used to exploit protein production and replication of the modified host bacteria. Another
15 factor involved in the amount of protein produced is the amount or efficiency of protein production in each bacterium. The proportion of materials produced by heterologous DNA to that produced by the host's own DNA is typically the same from cell to cell and from
20 generation to generation.

Most plasmids exist in only one copy per bacterial cell. Some plasmids, however, exist in higher copy number than one. For example, the plasmid ColE1 typically exists in 10 to 20 plasmid copies per
25 chromosome in E. coli. With certain plasmids it is possible to increase the relative proportion of plasmid DNA in the cell by adding a protein synthesis inhibitor such as chloramphenicol or spectinomycin. Of course, this technique does not assist in the accumulation of
30 protein, if that is what is desired. Because of the presence of inhibitor, only the DNA yield will be higher.

Protein production may be enhanced by the use of so-called super promoters which provide for extremely high levels of protein expression by increasing levels
35 of messenger RNA. Such a technique has limitations, however, in the maximum rate at which the cellular



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machinery can operate.

Work at the University of California has succeeded in isolating mutants of plasmids wherein copy number is much greater than normal. For example ColE1 mutants have been isolated wherein plasmid copy number exceeds 10 to 20 copies per chromosome. However, use of such mutants to produce protein from exogenous DNA may frequently result in failure because of the existence of numerous deletions at important regions in the plasmids.

It is an object of the present invention to provide an improved cloning vector for recombinant DNA production of protein.

Another object of the invention is to provide an improved plasmid capable of existing in high copy number and of giving correct expression of protein.

A further object of the invention is to provide an improved method for obtaining high level protein production from heterologous DNA inserted in plasmid cloning vectors.

Other objects of the invention will become apparent to those skilled in the art from the following description, taken in connection with the accompanying drawings wherein:

FIGURE 1 is a schematic diagram of a plasmid pBGP120;

FIGURE 2 illustrates a copy number mutant plasmid derived from the plasmid of FIGURE 1 wherein a deletion has occurred; and

FIGURE 3 is a schematic diagram of the plasmid constructed in accordance with the invention.

Very generally, the plasmid of the invention comprises a sequence of DNA containing a site 11 for replication origin, a site 12 for formation of the replication primer strand, a site 13 for the initiation of readthrough expression, and a section 15 of heterologous DNA downstream from and under the control



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of the expression initiation site and encoded and in the proper reading frame to produce a desired protein. The sequence further has a disabling configuration which prevents readthrough transcription into the replication
5 primer strand gene. The result is a plasmid of high copy number capability which is stable in that deletions are substantially avoided.

In constructing the plasmid of the invention, a plasmid is selected which is useful as a cloning
10 vector. One such plasmid is shown and described by O'Farrell, P. H., Polisky, B., and Gelfand, D. H. in J. Bacteriology 134 (2):645 - 654 (1978). This plasmid contains a functional portion of the lac promoter and lac operon indigenous to the host bacteria, terminating
15 in an EcoRI restriction enzyme site 14 in the beta-galactosidase gene of the lac operon. This portion may be linked at the restriction site 14 to a heterologous gene oriented in the same orientation and having the same reading frame such that readthrough can
20 occur from the lac operon into the heterologous gene in the same reading frame.

This plasmid (pBGP120) exists in high copy number levels, approximately 10 to 20 plasmids per cell chromosome. Plasmids capable of existing at even higher
25 copy numbers are selected by typical prior art techniques. Such techniques include titrating levels of a cloned product produced by the plasmid DNA.

It has been concluded that it is typically a repressor produced by the repressor gene 16 of the
30 plasmid itself which keeps the copy number down, rather than some positive control which provides a stimulus that maintains an elevated copy number. The present invention is preferably applied to a plasmid where a negative control system of this type is present. In
35 this connection, reference is made to the work on PSC 101 chimeras by Cabello, P., Timmis, K. and Cohen, S.N.,



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Nature 259:285-290 (1976). Typically, several successive screening procedures may be required to select the high copy number mutants. In other words, it is important to the invention that the characteristic of high copy number is indigenous to the plasmid itself, and is not the result of any function uniquely possessed by a particular host bacteria. In any case, the selected plasmid has an altered repressor gene 16' (FIGURE 2) leading to high copy number, i.e., in excess of 10-20 per chromosome.

It has been discovered that plasmids thus selected, even though existing in high copy number, almost invariably undergo undesirable deletions of substantial portions of the plasmid (i.e., are unstable). In fact, loss of critical portions of the plasmid, such as the promoter which promotes translation of the inserted heterologous DNA, results in failure of the plasmid to produce the protein desired. The deleted form of the high copy number plasmid is shown in FIGURE 2. It may be seen that with the lac promoter and much of the lac operon missing, DNA inserted at the EcoRI site 14 may be replicated, but transcription can no longer be controlled from the lac promoter.

Replication does not begin spontaneously from the origin of plasmid replication 11 shown in FIGURE 1. Rather, to begin replication it is necessary for a replication primer RNA strand to be generated. Transcription of this RNA primer strand begins at the origin 18 of the replication primer strand which is located between the EcoRI site 14 and the repressor 16. It is transcribed counterclockwise on the outer DNA strand 12 and, in the normal situation, stops at the origin of plasmid replication site 11. In reality, however, a large number of RNA primer strands are made which extend beyond the origin site 11. For replication to take place, these RNA primer strands must be



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processed to trim the strands and delete the extraneous material.

Recent work has shown that the repressor 16 is a segment of RNA which prevents the processing of the RNA replication primer strands to trim the excess RNAs from the strands. Accordingly, replication is unable to originate at the origin site 11. This repressor RNA is made from the inner strand as viewed in FIGURE 1 in a clockwise direction, shown by the arrow 20. In the case of the high copy number mutant, this short RNA repressor has a single base mutation which, it may be hypothesized, prevents the folding of the repressor RNA in such a way as to block the processing of the excessively long primer strands.

In such high copy number mutants, it is postulated that deletions in the plasmids occur as a result of readthrough transcription initiated from the lac promoter (under cyclic AMP control) in a counterclockwise direction as viewed in FIGURE 1 into and through the replication primer strand. The cell either loses the plasmid by not replicating it or the cell turns off the lac promoter by appropriate deletions.

The direction of transcription of the lac promoter is shown in FIGURES 1 and 3 by the arrow 19. The transcription of the primer strand is in the same direction as lac and is shown by the arrow 22. Copy number alone is not enough to produce the instability, because the mutant is quite stable (i.e., full size) in cells where low levels of lac transcription occur.

So, with the above model in mind, a new improved approach can be utilized to generate stable, full-sized mutants. The new method involves termination of readthrough transcription from the regulatory and heterologous gene sequences by placement of a suitable transcriptional "stop" signal after the inserted heterologous gene. This can be done using known



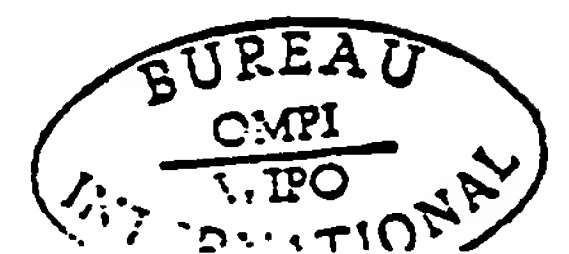
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techniques with appropriate restriction enzymes and ligase. See generally, Gentz, R., Langner, A., Chang, A., Cohen, S., and Bujard, H., Proc. Nat'l. Acad. Sci. USA 78:4936-4940 (1981).

5 Suitable transcriptional "stop" signals include the major terminator of the bacteriophage fd genome. The nucleotide sequence of bacteriophage fd DNA is known. See Beck, E., Sommer, R., Auerswald, E. A., Kurz, Ch., Zink, B., Osterburg, G., and Schaller, H.,
10 Nucleic Acids Res. 5(12):4495-4503 (1978). FIGURE 3 illustrates placement of a suitable transcriptional "stop" signal when the lac operon is used to provide the regulatory mechanism for the inserted heterologous DNA.

 It is useful to have temperature-sensitive (Ts)
15 copy number mutants. That is because high level production of some protein products of heterologous DNA may have a deleterious effect on the host cells. In temperature-sensitive copy number mutants, there is a maintenance of low copy number when the host cells are
20 grown at a "permissive" temperature (usually relatively lower). When the product of the heterologous DNA is desired in quantity, the temperature is shifted (usually increased). At this non-permissive or restrictive temperature, the mutation manifests itself and the
25 plasmid copy number increases, resulting in increased protein production from the plasmids.

 Such Ts mutants are rare, but can be found for almost any type of mutant by appropriate selection. First the location of the copy number mutant is
30 determined by fine-mapping with genetic recombination. Once the location is known, the spot is specifically mutagenized. This can be done by nucleotide alteration or by cutting out the region with restriction enzymes, strongly mutagenizing the fragment with some agents such
35 as bisulfite, hydroxylamine or ultraviolet radiation, and replacing the fragment. The resultant plasmids are



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transformed into cells that overproduce lac repressor. These are then plated over Xgal at permissive temperatures. Blue regions are removed from the culture. Then, at elevated temperatures, new blue areas
5 that appear are temperature-sensitive mutants which are picked.

These mutations are generally "recessive in trans.". That means, when a mutant and a normal plasmid are put in the same cell, the altered or "high copy
10 number phenotype" is masked.

The deleted form of the copy number mutant can exist in the same cell as the normal copy number parentaltpe plasmid. (This is not true of the non-mutant because of the property of incompatibility.
15 In spite of the similar mode of replication, this property is not manifested with the deleted form). When they are placed in the same cell, the copy number of both types of plasmids falls to normal (10-15). Thus, it can be concluded that a diffusable product - the
20 repressor - normally maintains the low copy number, but is absent or inactive in mutant form.

However, it is useful to have a copy number mutant that is dominant in trans. this allows a more generalized approach in that non-copy number mutant
25 plasmids with similar modes of replication can be grown to a high copy level in hosts carrying such a trans-dominant mutation.

It may be seen, therefore, that the invention provides an improved plasmid capable of existing and
30 replicating in high copy number and of giving correct expression or protein. By high copy number, it is meant typically in excess of fifty plasmids per chromosome and preferably in the range of one hundred to five hundred or greater. The plasmid thus enables one to obtain very
35 high level production of protein from heterologous DNA.

Various modifications of the invention in



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addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of
5 the appended claims.



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CLAIMS:

1. An improved DNA plasmid having a site for replication origin, a gene for formation of a replication primer strand, a site for the initiation of readthrough expression, a section of heterologous DNA
5 downstream from said expression initiation site encoded and in proper reading frame to produce a desired protein, and an altered repressor gene leading to high copy number replication, wherein the improvement comprises a suitable transcriptional stop signal located
10 after said heterologous DNA to prevent readthrough transcription into said gene for the replication primer strand.

2. A plasmid according to Claim 1 wherein said repressor gene is altered in such a way as to produce
15 defective repressor.

3. A plasmid according to Claim 1 wherein said repressor gene is altered in such a way as to produce no repressor.

4. A plasmid according to Claim 1 wherein said
20 expression initiation site comprises a site for the initiation of translation and at least a substantial portion of a gene which is indigenous to the host species of bacteria whereby readthrough can occur from said indigenous gene portion into said heterologous DNA.

25 5. A plasmid according to Claim 1 having a high copy number mutant repressor gene which is temperature-sensitive and trans-dominant.

6. A method for increasing the production of protein by heterologous DNA in plasmid cloning vectors,
30 comprising: selecting mutants of the plasmid having altered repressor genes leading to high copy number replication, inserting the heterologous DNA into the cloning vectors under the control of a promoter, and



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disabling readthrough transcription from the promoter into the replication primer strand gene of the plasmid cloning vector by interposing a suitable transcriptional stop sequence between said heterologous DNA and said replication primer strand gene.

7. A method according to Claim 6 wherein the copy number mutants are temperature-sensitive copy number mutants.

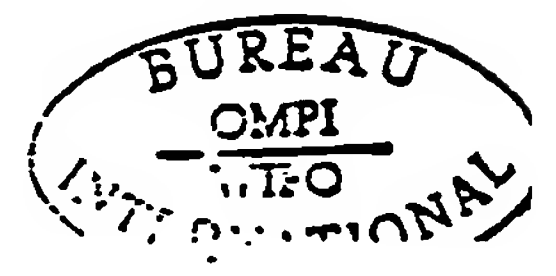
8. A method according to Claim 6 wherein the copy number mutants are selected in such a way that such mutants are dominant in trans.

9. A method for producing protein in a host microorganism, which protein is encoded by heterologous DNA, said method comprising utilizing a host microorganism containing plasmids in higher than normal copy number, each of said plasmids comprising: a site for replication origin, a site for formation of a replication primer strand, a site for the initiation of readthrough expression, a section of heterologous DNA downstream from said expression initiation site encoded and in proper reading frame to produce a desired protein, and an altered repressor gene leading to high copy number replication, said plasmid further having a suitable transcriptional stop signal located after said heterologous DNA to prevent readthrough transcription into said site for the replication primer strand.

10. A method according to Claim 9 wherein said repressor gene is altered in such a way as to produce defective repressor.

11. A method according to Claim 9 wherein said repressor gene is altered in such a way as to produce no repressor.

12. A method according to Claim 9 wherein said expression initiation site comprises a site for the initiation of translation and at least a substantial portion of a gene which is indigenous to the host

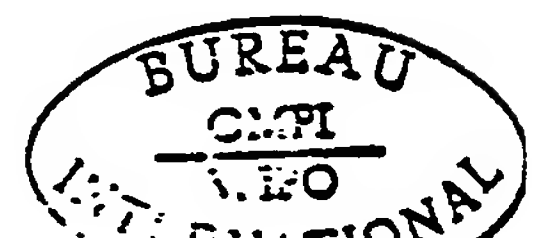


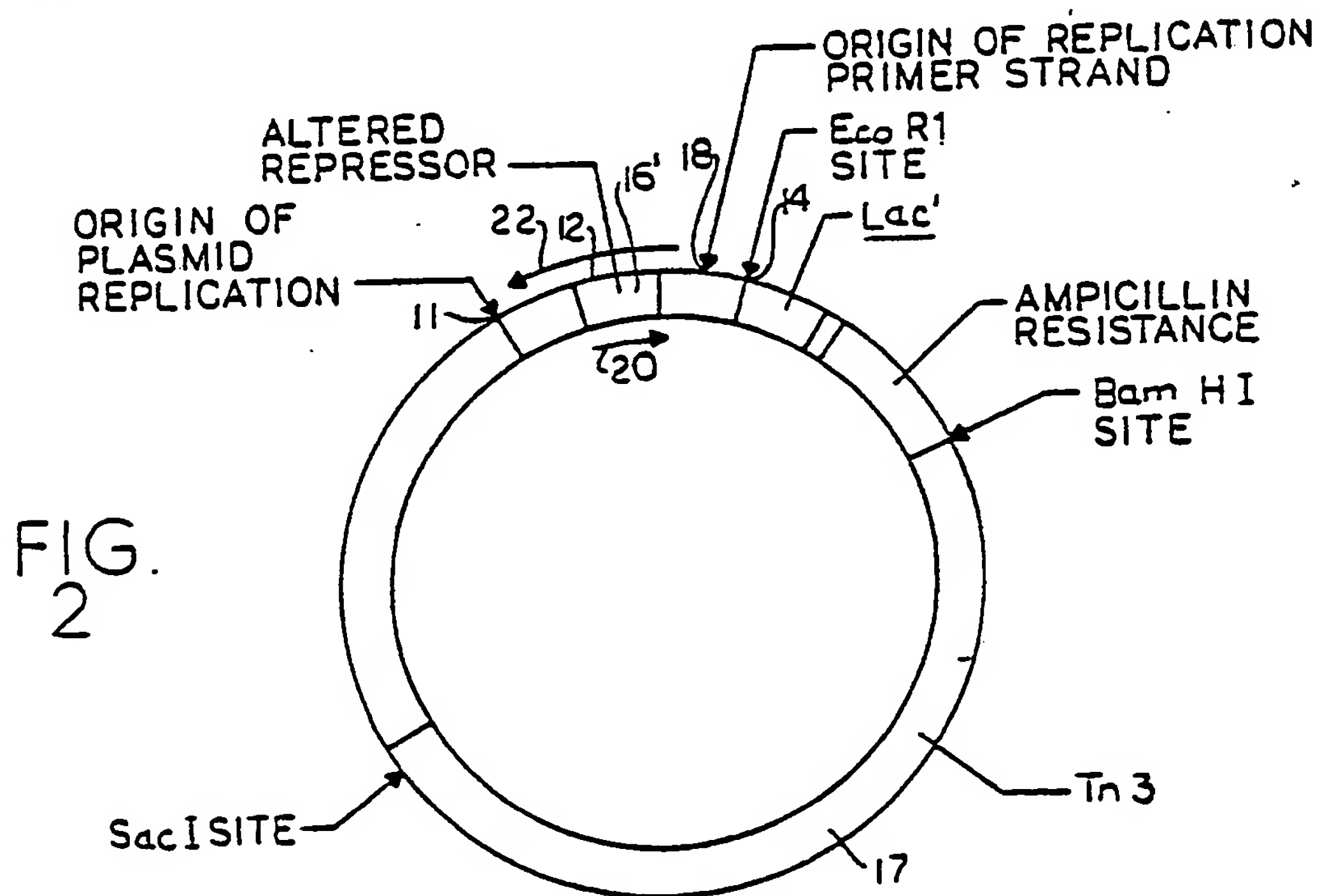
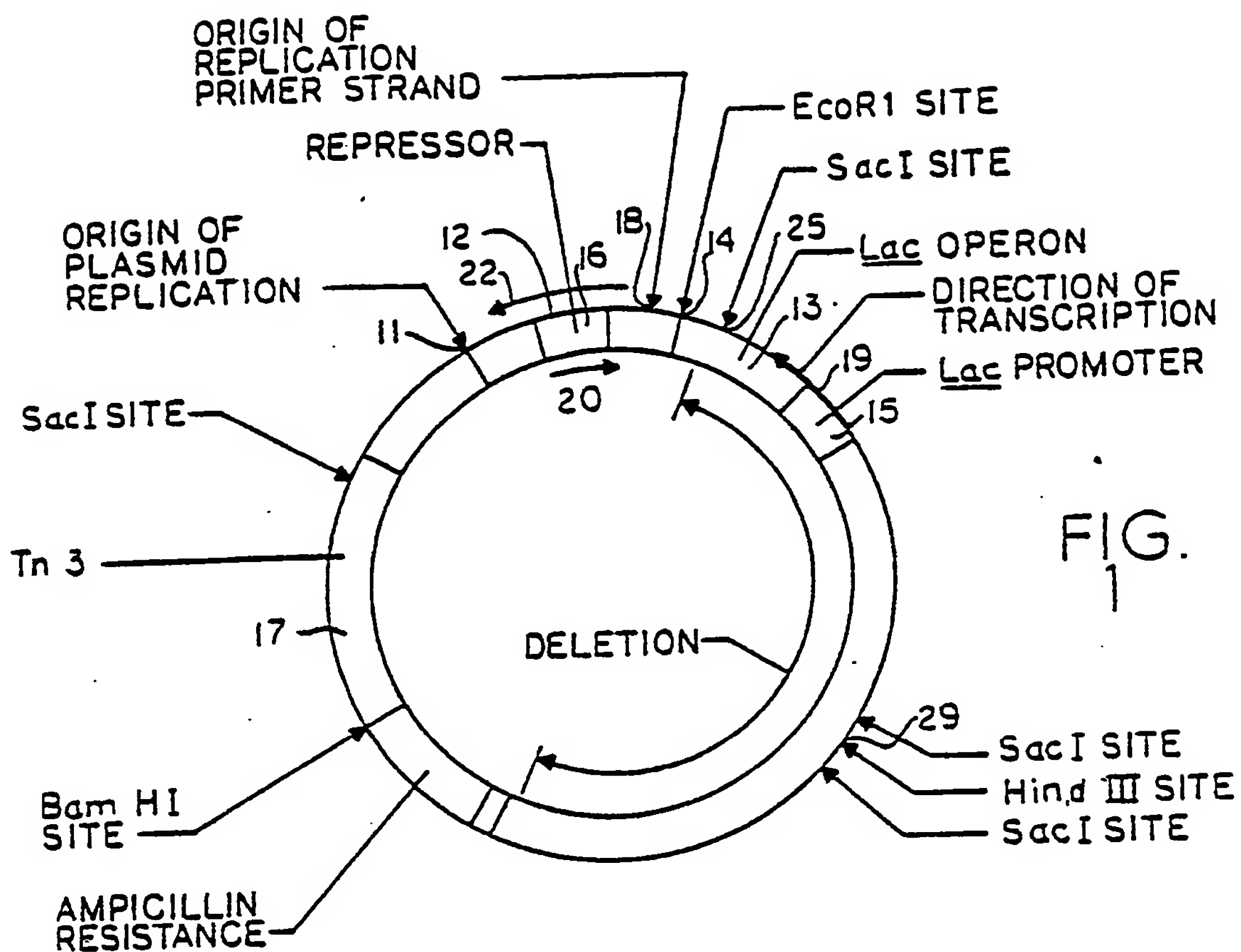
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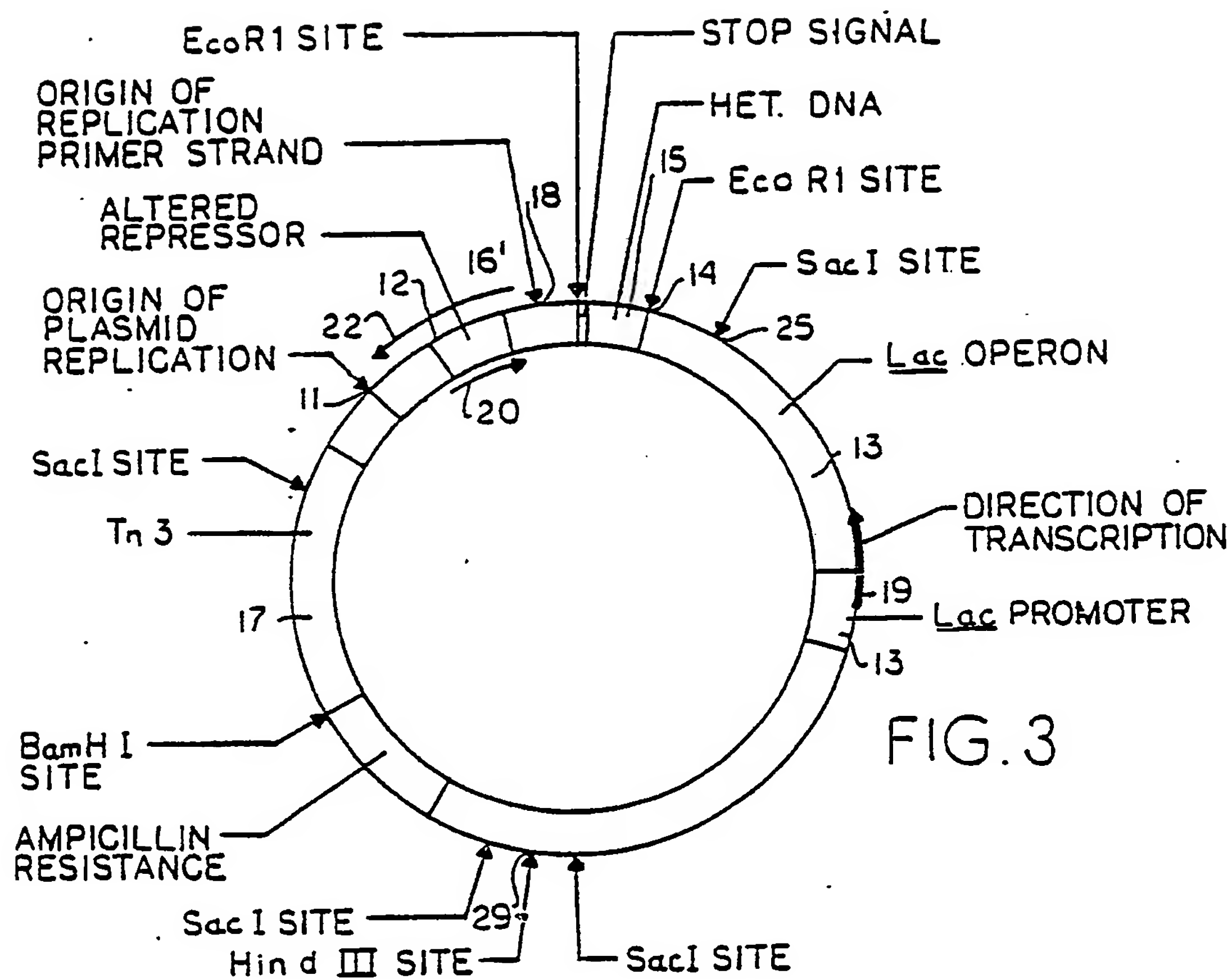
species of bacteria whereby readthrough can occur from said indigenous gene portion into said heterologous DNA.

13. A method according to Claim 9 having a high copy number mutant repressor gene which is
5 temperature-sensitive and trans-dominant.

14. A protein produced in accordance with the method of any of Claim 9-14.








INTERNATIONAL SEARCH REPORT

International Application No PCT/US82/00246

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC INT. CL. C12N15/00, 1/00; C12P 21/00, 21/02, 21/04; C07G 7/00 U.S. CL. 435/172, 317, 68, 70, 71; 260/112.5R, 112.5S		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/172, 317, 68, 70, 71 260/112.5R, 112.5S	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 14		
Category *	Citation of Document, 14 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X, E	US, A 4,322,499 PUBLISHED 30 MARCH 1982.	14
A	N, MOLEC. GEN. GENET. VOL. 178 PP 525-533 1980.	1-13
A	N, NATURE VOL 259 PP 285-290 JANUARY 29, 1976.	1-13
A	N, JOURNAL OF BACTERIOLOGY VOL 137 PP 635-643 JANUARY 1979.	1-13
* Special categories of cited documents: 19		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"I" document cited for special reason other than those referred to in the other categories</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> </div> <div style="width: 45%;"> <p>"P" document published prior to the international filing date but on or after the priority date claimed</p> <p>"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search :		Date of Mailing of this International Search Report :
03 JUNE 1982		22 JUN 1982
International Searching Authority :		Signature of Authorizing Officer :
ISA/US		 ALVIN E. TANENHOLZ

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹⁴

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-5 and 9-13 drawn to plasmids and methods of producing protein using said plasmid classified in 435/317 and 435/68,
 II. Claims 6-8 directed to methods of modifying a plasmid classified in 435/172 and
 III. Claim 14 drawn to a protein classified in 260/112.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

- ☒ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.